Multiplex Polymerase Chain Reaction Method for Differentiating Western and Northern Corn Rootworm Larvae (Coleoptera: Chrysomelidae)

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J. Econ. Entomol. 96(3): 669–672 (2003)

ABSTRACT Western corn rootworm, *Diabrotica virgifera virgifera* LeConte, and northern corn rootworm, *D. barberi* Smith and Lawrence, are sympatric species and serious pests of corn cultivation in North America. Comparison of nucleotide sequence of mitochondrial cytochrome oxidase I and II was used to design polymerase chain reaction (PCR) primers that discriminate immature stages of the two species based on differences in amplicon size. Multiplex PCR can be used to give a positive test for each species in a single amplification reaction. This provides a method to identify field caught larvae and facilitates investigations of larval interaction and competition between the species.

KEY WORDS *Diabrotica*, corn rootworm, species diagnostics, mitochondrial DNA

CORN ROOTWORM BEETLES of the genus *Diabrotica* are a major economic pest of corn (maize) production in the United States. Two species, western corn rootworm, *Diabrotica virgifera virgifera* LeConte, and northern corn rootworm, *D. barberi* Smith and Lawrence, have overlapping distributions and can often be found in the same fields in the central part of the country. In most of the corn belt, western corn rootworm is the predominant species, with northern corn rootworm distribution being spotty and variable from year to year. However, in the northwestern portion of the corn belt (South Dakota, southwestern Minnesota, and western Iowa) the northern corn rootworm has recently gained numbers and is often the predominant species (Ellsbury et al. 1998, Potter 2001). Although adults can feed on tassels, silks, and kernels, most crop damage results from larval root feeding. Add to this the fact that the larvae and other immature stages are morphologically inseparable, and examination of the factors involved in species competition becomes difficult. The insects are not only under ground but they look alike.

Attempting to correlate the level and nature of root damage to the ratio of adult emergence may not reliably reflect what is happening in the soil. Greenhouse studies have indicated that western corn rootworm larvae have a competitive advantage over northern corn rootworm larvae when both are present in the same pot (Piedrahita et al. 1985; Woodson 1994a, 1994b). In these studies, defined numbers of rootworm eggs of one species or different ratios of both species were introduced into pots with a single corn plant. The studies concluded that the presence of western corn rootworm larvae was detrimental to the survival of northern corn rootworm larvae. The sampling was different between the two projects. Piedrahita and coworkers examined preadults using an unpublished allozyme assay whereas Woodson counted adult emergence. Woodson also noted an intraspecific effect for both species in that overcrowding reduces the number of adults. A species difference in spatial distribution was observed by Piedrahita and coworkers. Western corn rootworm larvae seemed to cluster in the proximal part of the root zone. Northern corn rootworms were evenly dispersed by themselves, but in combination with western corn rootworms, the northern corn rootworms were pushed distally. Sutter and Hovland (1995) confirmed the clustering of western corn rootworm larvae near the base of the plant and the more peripheral distribution of northern corn rootworm larvae in field experiments. The recent ascendance of northern corn rootworm in South Dakota clearly demonstrates that western corn rootworm's competitive superiority does not apply in all situations.

The paucity of information pertaining to the interactions of larval northern corn rootworm and western corn rootworm with each other and with the corn root system is, in part, attributable to inadequate methods for scoring the species. It has previously been shown that both nucleotide sequencing and restriction enzyme digestion of polymerase chain reaction (PCR) amplicons can be used to differentiate between these species (Szalanski and Powers 1996, Szalanski et al. 2000), but these techniques are expensive or time-consuming. To study northern and western corn rootworms and their interactions, especially in field locations where both species are present, a more con-

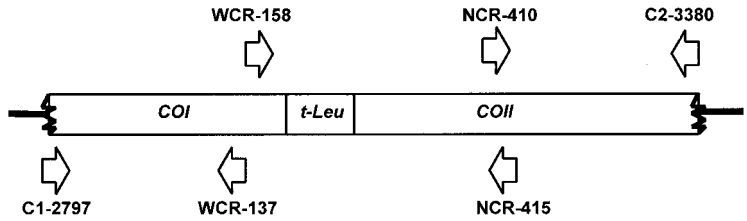

Fig. 1. Location of *Diabrotica* species-specific mtDNA primers. The relative positions and directions (arrows) of the PCR primers in the mitochondrial COI-COII region.

venient method was needed to identify the morphologically indistinguishable larvae. DNA sequence differences reported between northern corn rootworm and western corn rootworm in a small portion of the mtDNA (Szalanski et al. 2000) were exploited to design species-specific PCR primers and to develop a DNA-based assay that can determine the species of individual larvae.

Materials and Methods

Western corn rootworm and northern corn rootworm adults were from field collections at various locations in the central United States. Western corn rootworm larvae were from the colony at the Northern Grain Insects Research Laboratory (NGIRL), Brookings, SD. Northern corn rootworm larvae were also from NGIRL and were acquired from eggs laid in the laboratory by field collected adults. Unknown corn rootworm larvae were obtained from field plots at NGIRL. Individual corn root systems were dug and brought into the lab. The roots were dried above a water trap (Fromm et al. 1998) and larvae recovered were frozen at −70°C.

Total DNA from adults and some larvae of each species was prepared using a high salt method (Cheung et al. 1993). To shorten the preparation time to examine larger numbers of larvae collected in field, some larval extracts were prepared with an alternate procedure. A single larva was placed in a 500-μl plastic tube with 25 μl of homogenization buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% Nonidet, 100 μg/ml Proteinase K). The larva was crushed with a plastic pestle and the mixture was heated to 95°C for 3 min then centrifuged in the cold for 5 min at 12,000 × g with the supernatant removed to a fresh tube. Five microliters of the resultant solution was used in a 50-μl PCR. PCR reaction mix consisted of 5 μl 10× PCR Buffer II (Perkin Elmer), 4 μl 25 mM Mg Cl, 1 μl each of 10 mM nucleotides, 0.5 μl Amplitaq Gold polymerase (Perkin Elmer), 0.5 μl each of 100 μM primers, and sterile water to volume. The primers used included: C1–2797 (C1-J-2797, 5′-CCTCGACGTTATTCAGATTACC-3′, Simon et al. 1994); C2-3380 (C2-N-3380, 5′-TCAATATCATTGATGACCAAT-3′, Taylor et al. 1997); WCR-137 (C1-N-2950, 5′-AGCTGGAGGGTTAAATTGTAG-3′); WCR-158 (C1-J-2991, 5′-GAACACAGATATTCTGAAGTG-3′); NCR-410 (C2-J-3250, 5′-TTTTTGCACCGAAATCTATTA-3′), and NCR-415 (C2-N-3231, 5′-CCTTCTAATAGATTTCGGTGC-3′). The four-digit numbers demark the 3′ end of the primers in the *D. yakuba* mtDNA sequence (Clary and Wolstenholme 1985). The optimal annealing temperature was determined for each primer set by conducting PCR in the temperature gradient block of the Stratagene Robocyler Gradient 40 thermocyler (Stratagene, La Jolla, CA). The temperature gradient was from 46–60°C in two-degree steps. The final amplification conditions chosen were 35 cycles of 94°C 1 min, 50°C 2min, and 72°C 3 min in either a Perkin-Elmer 480 or the Stratagene Robocyler Gradient 40 thermocyler. Amplicons were visualized by running 20 μl of PCR product on 1% agarose gels with ethidium bromide. Gel photographs were scanned to generate the negative images of Fig. 1. When testing unknown larvae and extracts from known western corn rootworm and northern corn rootworm were included with each PCR reaction batch.

GenBank accession numbers for the western corn rootworm and northern corn rootworm sequences are AF195196 and AF195197, respectively. The sequences were aligned using AlignX (Vector NTI Suite, http://www.informaxinc.com).

Results and Discussion

A segment encompassing the 3′ portion of the mtDNA cytochrome oxidase I (COI) gene, tRNA leucine, and a 5′ portion of the cytochrome oxidase II (COII) genes from western corn rootworm and north-

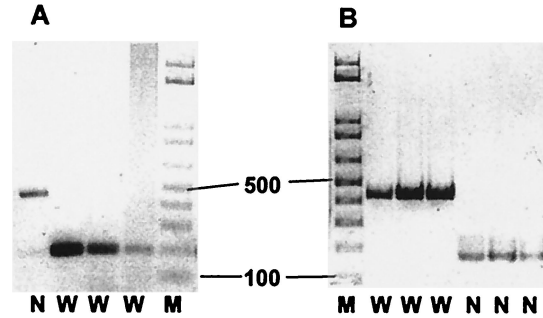

Fig. 2. PCR products with *Diabrotica* species-specific primers. (A) Primers WCR-137, NCR-415, C1-2797. (B) Primers WCR-158, NCR-410, C2-3380. N = northern corn rootworm; W = western corn rootworm; M = molecular marker (size in base pairs). Figures are negative images of ethidium bromide stained gels.

ern corn rootworm was amplified and sequenced as previously described (Szalanski et al. 2000). The western corn rootworm (WCR) and northern corn rootworm (NCR) sequences were aligned and examined for mismatches that reflected either substitutions or deletions. The mismatches were exploited to design primers that were unique to each species. Two primers, one from each strand, WCR-137 and WCR-158 were made from the WCR COI gene. Similarly two primers were made from the northern corn rootworm COII gene, NCR-410 and NCR-415. The new primers were tested individually in combination with common primers, either C1-2797 or C2-3380 depending on their orientation (Fig. 1). Based on the sequence, the expected sizes of the amplicons are 423 bp (WCR-158 + C2-3380), 171 bp (NCR-410 + C2-3380), 195 bp (WCR-137 + C1-2797), and 424 bp (NCR-415 + C1-2797). Proper sized PCR products were obtained using conspecific DNA, whereas no product was obtained with template from the other species, i.e., no false positives were observed using known DNA. Each WCR primer paired with a common primer will only amplify western corn rootworm DNA and similarly NCR primers will only amplify northern corn rootworm DNA.

Each of the four species-specific primers was tested for optimal annealing performance in a 46°–60°C temperature gradient with 2°C intervals. Two of the primers successfully amplified at all eight temperatures, whereas the other two primers performed poorly at 58°C and 60°C; 50°C was chosen as a middle annealing temperature suitable for all the primers.

Because the positive amplification products are a different size for each species and can be easily distinguished on gels, it has been possible to use multiplex PCR (i.e., two or more species-specific primers and a common anchor in the same reaction). Multiplex PCR using the three primers, WCR-137, NCR-415, and C1-2797 (Fig. 2A) and the three primers, WCR-158, NCR-410, and C2-3380 (Fig. 2B) produced the expected amplicon sizes and validates the multiple primer approach.

The shorter DNA preparation protocol proved to be effective for the larvae. It is more of a crush-and-go procedure that eliminates time consuming precipitation, centrifugation and rehydration steps. In the context of determining the species for a large number of larvae collected in connection with a distribution or competition studies, simplifying the extraction protocol is advantageous. The drawbacks to the shorter DNA prep is lower yield of PCR product and the DNA appears to degrade much more quickly. Many samples kept in the refrigerator for a couple weeks were inadequate for amplification. This could be a disadvantage if the PCR needed to be repeated.

Western corn rootworm in the United States has very little genetic polymorphism across its geographic distribution (Szalanski et al. 1999). By contrast, northern corn rootworm is genetically diverse with polymorphism both at the local population level and on a broader geographic scale. To check whether the primers reacted differently depending on the geographic origin of the template, the primer combinations were tested with DNA from adults and larvae of 20 western corn rootworms from South Dakota, North Dakota, and Indiana and adults of 38 northern corn rootworms from North Dakota, South Dakota, Wisconsin, Iowa, Pennsylvania, and five sites in Illinois. The western corn rootworms were from locations previously shown to have extremely low mtDNA polymorphism (Szalanski et al. 1999). The northern corn rootworm samples are from both sides of a major population genetic boundary located in Illinois (unpublished). All 58 insects produced PCR products of the anticipated size for their species. Figure 2B has examples of western corn rootworm adults from the three locations and northern corn rootworm adults from three of the ten locations. The amplification was equally successful regardless of the geographic origin of the DNA.

In the context of other studies >1000 unknown rootworm larvae have been examined using the abbreviated extraction procedure and multiplex primers. Approximately 10–12% of the unknown extracts could not be scored for either species because no amplification was detected. One possible reason for this would be unexpected mtDNA polymorphism in the samples so that the species-specific primers no longer bind to the DNA sufficiently to allow amplification. Another possible reason for the number of negatives is that the extracts did not contain enough intact DNA for amplification. This could be the result of improper handling of the larvae as they were collected or inconsistencies in the DNA extraction procedure. Of the DNA samples that failed to amplify anything with the species-specific primers, 37 were retested with conserved sequence insect mtDNA primers from the COI, COII, or 16S rRNA (large ribosomal RNA) genes. Only one of these generated a PCR product, which argues strongly against the unknown polymorphism possibility.

Employing the principles of integrated pest management to control corn rootworms while reducing pesticide loads makes it imperative that we have some knowledge of the dynamics of their interspecies interaction. These primers provide a convenient way to identify individual larvae without resorting to the more time consuming restriction fragment-length polymorphism analysis. The approach is equally applicable to other life cycle stages, such as pupae. Early experiments using this technique with material gathered from the field have already given evidence that the ratio of northern corn rootworm to western corn rootworm in emerging adults may not be the same as the ratio of larvae in the root ball (unpublished data). This should be an important new tool for examining the species competition and preferred larval locations in the corn root system.

Acknowledgments

Thanks to S. Degrugillier, L. Hammack, D. Horvath, L. Chandler, and A. Szalanski.

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Received for publication 9 May 2002; accepted 9 December 2002.